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MORPHOLOGY AND GENETICS OF REJECTION REACTIONS BETWEEN OOZOOIDS FROM THE TUNICATE *BOTRYLLUS SCHLOSSERI*

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ABSTRACT

Botryllus rejection reactions were followed in pairs of oozooids placed together immediately after initiation of metamorphosis. Within twelve hours, both compatible and incompatible oozooid pairs underwent tunic fusion and initiation of ampullar tip-to-side contact. Vascular fusion followed within two days between compatible pairs, while the fusion sequence was interrupted in the incompatible pairs by a rapid cytotoxic rejection response. Events occurring within and outside the ampullae in rejections were effector responses whose consequences were separation of the ampullae and isolation of the involved tissues from the bodies of the oozooids. Genetics experiments suggested that the four distinct types of rejection reflect a hierarchy of histoincompatibility in this system.

INTRODUCTION

Recent interest in colonial tunicates has centered around the phenomenon of *colony specificity*, which is the capacity for self-nonself distinction leading to fusion or rejection between colonies. In *Botryllus*, this histocompatibility discrimination is controlled by a single multiallelic Mendelian locus (Oka and Watanabe, 1960; Sabbadin, 1962) that resembles loci within the vertebrate major histocompatibility complex, or MHC (Scofield *et al.*, 1982a). We have undertaken studies to determine whether genes controlling allogeneic recognition in *Botryllus* are homologous to those within the MHC. To complement our molecular studies, we have examined *Botryllus* rejection responses in live preparations of rejecting oozooids, using differential interference contrast (Zeiss-Nomarski) microscopy.

Botryllus colonies are clones of individuals, or *zooids*, enclosed in a common tunic. Each zooid is parabiosed to all the others through a colonial vascular network that is terminated at the colony periphery by bulbous ampullae. Tanaka and Watanabe (1973) have shown that fusions and rejections between colonies are contact responses between their ampullae. All the individuals in a colony arise by budding from the "founder" individual, or oozooid, that is established by metamorphosis of a swimming tadpole-like larva. Oozooids possess eight microampullae, and paired oozooids undergo vascular fusions and rejections similar to those occurring between grown colonies (Scofield *et al.*, 1982a, b).

When the separated growing edges of the same *Botryllus* colony meet, the tunic (test) dissolves, and the opposite ampullae interdigitate to form tip-to-side contacts (Tanaka and Watanabe, 1973). This sequence of events is part of the morphogenetic "program" that establishes blood flow between the contacted blood vessels (Katow

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and Watanabe, 1980). Fusion also proceeds, without interruption, between colonies sharing at least one allele at the fusion locus (Oka and Watanabe, 1960). For colonies sharing no fusibility alleles, however, the fusion sequence is aborted after ampullar contact, and is followed by a cytotoxic rejection (Tanaka and Watanabe, 1973). Recently, Taneda and Watanabe (1982a, b, c) firmly established that the allorecognition elements that allow fusion, or cause rejection, are humoral and cellular elements in the blood. To outline the sequence of cellular events that follow allorecognition and lead to a completed rejection response, we followed rejections *in vitro* in paired oozooids.

In other invertebrates (Ivker, 1972), as in vertebrates (Götze, 1977), polymorphic histocompatibility gene systems show a hierarchy, manifested by differences in the timing and severity of rejection responses that depend upon particular alleles possessed by the contacted cells. To determine whether such a hierarchy exists for *Botryllus* fusibility alleles, we subjected colonies to different kinds of genetic crosses, and scored rejection "types" for the offspring.

Materials and Methods

Colonies of *B. schlosseri* were gathered from the Eel Pond in Woods Hole, Massachusetts, and maintained with constant aeration in beakers of filtered sea water. Tadpole larvae were gathered by placing coverslips along the waterline inside the beakers, where they attached and underwent metamorphosis to form natural pairs.

For time-lapse observations, coverslips carrying oozooid pairs were inverted over a drop of sea water on a glass microscope slide. Observations were made using Zeiss-Nomarski optics. Between observations, the coverslips were cultured in their original beakers.

Genetic crosses were carried out in the same beakers that were used to culture single colonies. Three sets of experiments were done. First, colonies already carrying developing embryos ("wild-fertilized" colonies) were collected and cultured until the developing tadpoles hatched and metamorphosed to form oozooid pairs. For "defined" crosses, pairs of colonies were placed in beakers, where eggs of one colony were fertilized only by sperm from the crossing partner. For "self" crosses, colonies were isolated and self-fertilizations were allowed to proceed (Scofield *et al.*, 1982a). After each cross, colonies containing fertilized eggs were cultured in isolation until the F_1 larvae hatched.

Results

The thin oozooid preparations allowed easy visualization of ampullar junctions under the microscope. Rejections and fusions occurred readily between paired oozooids within two days of hatching and metamorphosis. Within 12–24 hours of contact, blood flow was established and connecting vessels formed between fusible oozooids (Fig. 1A). Likewise, rejection reactions usually were completed by one day after initiation of ampullar contact. The characteristic feature of oozooid rejections was a bright golden-brown necrotic zone (Fig. 1B).

Figure 2A shows a normal ampulla photographed at its point of attachment to an inverted glass coverslip. The surfaces of the "tip" cells are smooth, and the surrounding tunic contains only the interconnected "test cells" (Fig. 2A). In rejecting oozooid pairs, by contrast, the ampullae and the surrounding tunic showed striking alterations. After a period of tip-to-side contact, ampullar reseparation was followed rapidly by movement of blood cells through the "tipping" ampullar tip into the tunic (Fig. 2B). Closer examination of the cytotoxic mass revealed concave "holes" in the

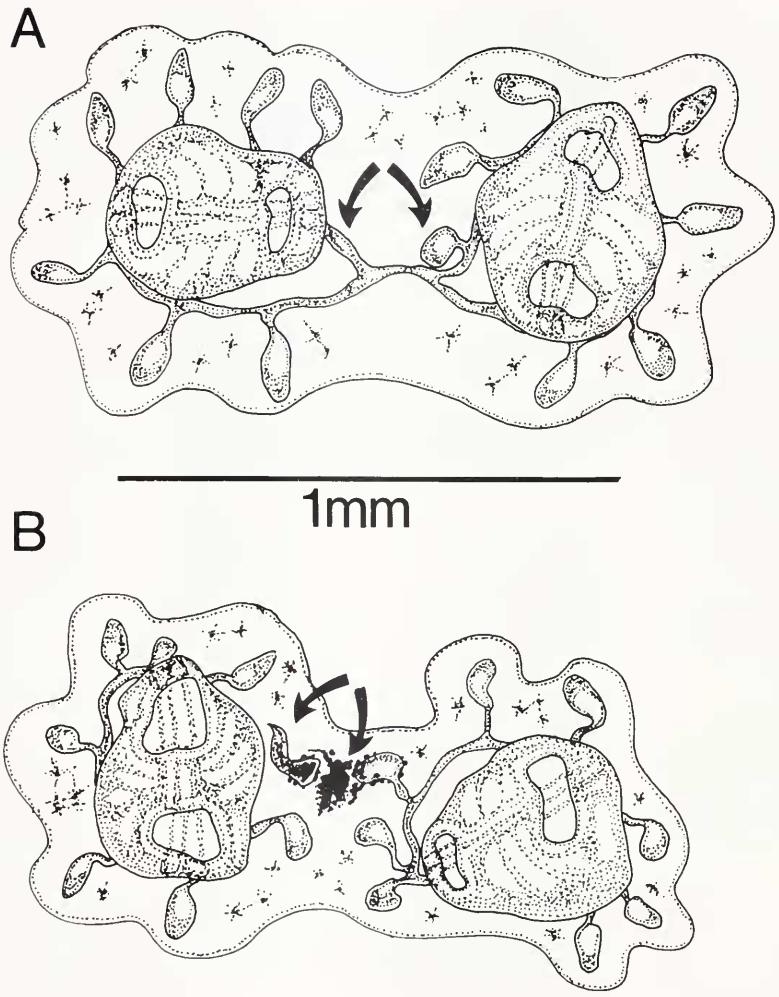
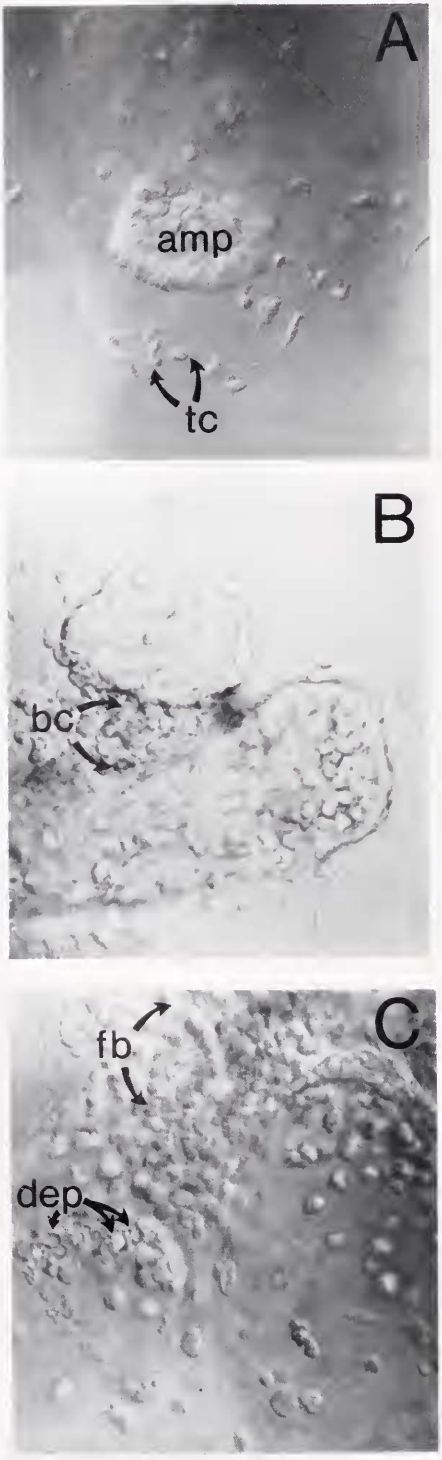


FIGURE 1. Fused and rejected *Botryllus* oozooid pairs. A. Fused oozooids, showing the connecting blood vessels (arrows) at the site of a prior tip-to-side contact. B. Rejected oozooids, showing the necrotic zone and an autoamputated ampulla (arrows).

tip cells (Fig. 2C). In some pairs, blood flow inside the involved ampullae slowed to a stop. Emboli broken from these clotted masses frequently plugged the proximal end of the ampulla (Fig. 3A) at sites where amputation eventually occurred (see below). Examination of the blood cells released into the tunic revealed that the first to appear there had the distinct berry-like appearance of morula cells (Fig. 3B). Their vacuoles had turned a dark brown. After deposition into the tunic, morula cell disintegration was accompanied by condensation of fibers at the site (Fig. 2C).

Other morula cells, morula cell precursors (signet-ring and compartment cells), and granular amoebocytes were shed into the tunic as the ampullae retreated from the contact point. These, however, remained transparent by transmitted light (Fig. 2C). Some developed processes and moved away from the rejection site, while others contributed to the cytotoxic mass (Fig. 2C). It is clear from Figures 2 and 3 that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rejection reactions following allogeneic contacts in *Botryllus* are extremely destructive to surrounding tissues.

A surprising finding was that different oozooid pairs from the same hatching gave very different rejection responses. Although necrotic regions always appeared between rejected oozooids, a striking difference in timing of rejection events and gross appearance of the cytotoxic lesion became evident after examination of many pairs. The time between establishment of ampullar junctions and rejection was highly variable, ranging between about 30 minutes and approximately 12 hours. Completed rejection responses could be placed into one of four categories (1–4; Fig. 4, Table I) that were distinguished easily by reflected light. The several forms taken by oozooid rejections in this study appear in Figure 4.

"Type 1" rejections showed very slight bleeding from the "tipping" ampulla following ampullar reseparation. In most instances, careful inspection of the retreating ampullae was necessary to visualize the few golden-brown cells bled from their tips. This sometimes was accompanied by visible "sticking" of the rounded tip cells onto the "side" ampulla at the prior contact site (Fig. 4A). The "type 2" response was a more extensive bleeding of the "tipping" ampulla, again with the ampulla itself remaining sealed and generally intact. In both these types of bleeding responses, the characteristic brown color reaction was seen in the rejection lesion, but not within the ampullae.

The third type of rejection (type 3) was autoamputation (Fig. 4B), occurring with or without ampullar bleeding from the tip. For these rejections, the entire amputated ampulla, and its contents, turned brown. The rejection type "4" was ampullar disintegration, where the ampullar contents and epithelium became part of the colored rejection mass (Fig. 4C).

It appeared that these rejection types represented a continuum of responses, with the differences being a function of the extent to which the ampullae moved through the fusion sequence before it was aborted (for example, a brisk response to a rapid allorecognition might account for both the minor and more extensive bleeding responses, while more extreme ampullar reactions—amputation or disintegration—would result from more extensive mixing of allogeneic blood elements).

Because the distinct responses seemed to reflect different thresholds for "effective" allorecognition, we proposed that these differences actually reflect a nested hierarchy of histoincompatibility for the many fusibility alleles. If so, different oozooid pairs with the same combinations of fusibility haplotypes would be expected to give the same kind of rejection response. An oozooid microassay (Scofield *et al.*, 1982a) was used to test this hypothesis with different genetic crosses (Fig. 5).

Since wild colonies usually are heterozygotic at one Mendelian gene for fusion at which there are many alleles (Oka and Watanabe, 1960), any given colony can be named AB at this locus (Fig. 5, top) and the diploid progeny of that colony will be A or B with respect to the maternal fusibility allele (Scofield *et al.*, 1982a). Certain predictions can be made regarding genotypes of F_1 oozooid pairings that give rejections.

FIGURE 2. Anatomy of normal ampullae and of ampullae participating in rejection reactions. A. Normal ampullar tip (*amp*), surrounding tunic, and test cells (*tc*) in a *Botryllus* oozooid. The ampullar tip cells are columnar, vacuolated, and tightly interconnected. B. Ampullar withdrawal following a rejecting tip-to-side contact. Blood cells (*bc*) can be seen moving from the "tipping" ampulla (top) into the tunic where they undergo cytotoxic interactions and cause fiber deposition. The "side" ampulla (bottom) also is filled with clumped blood cells. C. Changes in ampullar tip cells after a rejecting tip-to-side contact. Rounded "holes" or depressions (*dep*) appear in the tip cells of the interacting ampullae. The brown fibrous barrier deposited by blood cells in the tunic (*fb*) appears at the top. A and B, ×500. C, ×1000.

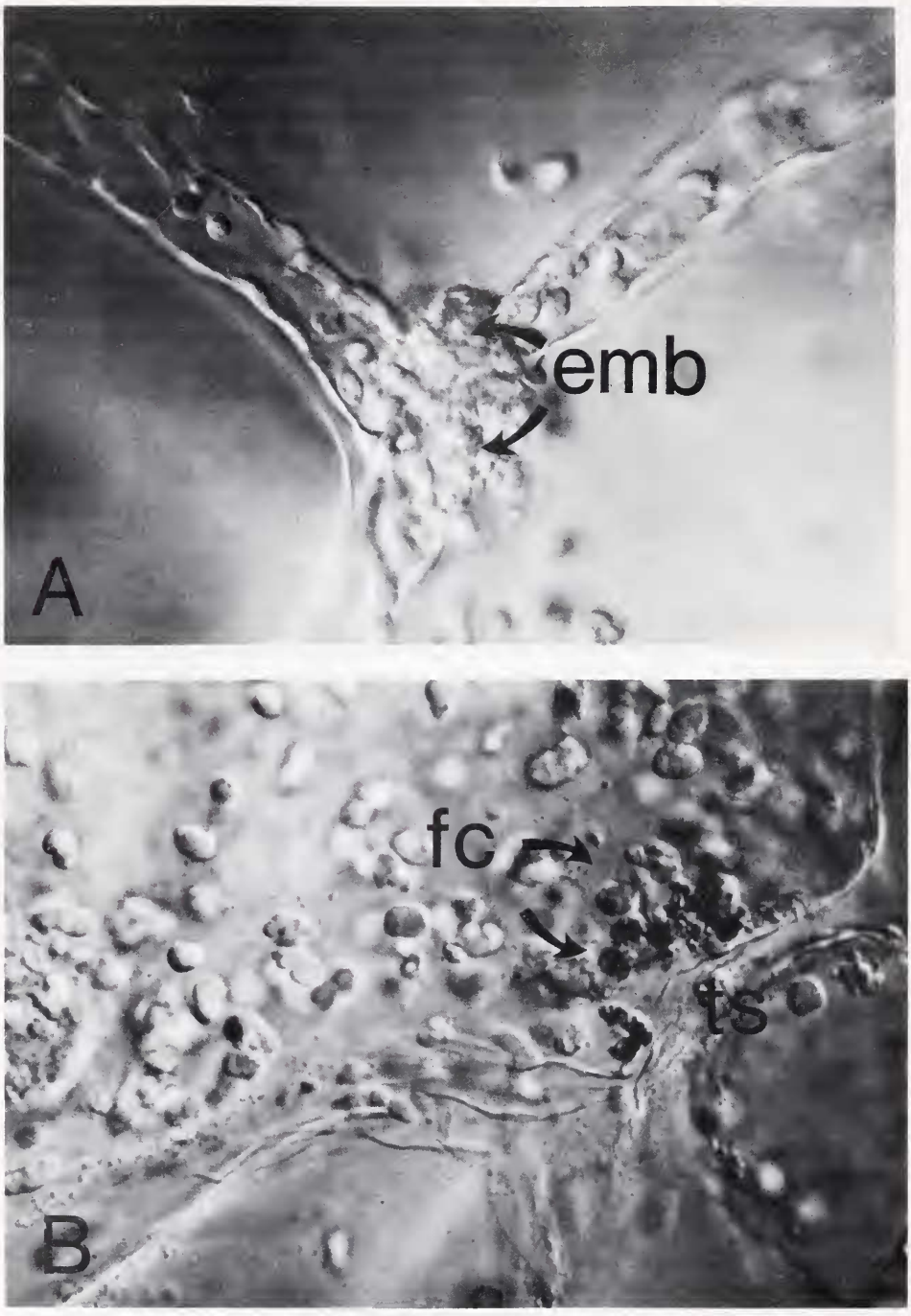

FIGURE 3. Effector responses in oozooid blood vessels and tunic after a rejection reaction. A. Embolus (*emb*) of clotted blood cells and fibers preventing backflow of blood through the proximal end of an ampulla participating in a rejection response. B. Ferrocytes (*fc*), deposited into the tunic from the ampullar tip at the right, have vacuoles which have turned dark red-brown. ×1000.

For example, if a colony is fertilized in the natural environment by sperm from many different colonies, the randomly combined A and B oozooids yield 50% fusing and 50% rejecting pairs (the chance that any two share a paternal allele is small, Fig. 5, left). Because many different sperm fertilize in such "wild" crosses, rejections between the progeny oozooids involve many different allelic combinations (there are 50–100 fusibility alleles in natural populations—Schlumpberger and Scofield, unpub.). If rejection type depends upon fusibility alleles, then the pairs of rejected progeny from these wild crosses should show some frequency distribution of all four rejection types (Fig. 5, left).

If the same maternal AB colony is crossed by only one other colony, CD (Fig. 5, center) then only C and D sperm fertilize; the F_1 progeny are of four types, and that 25% of the progeny pairs which reject are of only two haplotypic combinations. Thus only one or two different rejection types should be represented in the paired progeny of a "defined" cross. If the AB colony is *self*-crossed, on the other hand, the rejected 12.5% of the experimental pairs are of only one allelic combination (Fig. 5, right); thus only one rejection type should be found.

Results from these experiments appear in Table I. As expected, wild-fertilized colonies yielded oozooids which, when paired, showed 50% fusions and 50% rejections (numerical data not shown; Fig. 5, left). Rejection types were distributed fairly evenly over all four categories. The defined crosses, on the other hand, hatched progeny whose pairs gave 75% fusions and 25% rejections (Fig. 5, center). The rejected pairs from these crosses generally showed only one or two rejection types; exceptions were seen only in the progeny of two crosses (defined crosses 6 and 7) where ampullar amputation in some pairs was accompanied by bleeding from the tips. Self-crossed colonies produced progeny whose pairs gave very few rejections, both because they represent only 12.5% of the total pairs (Fig. 5, right) and because inbreeding depression reduces the total number of hatched larvae (Sabbadin, 1971; Scofield *et al.*, 1982a). However, those rejections were all of one type in three experiments (Table I).

Discussion

Fusions and rejections between *Botryllus* oozooids appear to be similar to those occurring between adult colonies (Tanaka and Watanabe, 1973; Katow and Watanabe, 1980). For incompatible pairs, the ampullae move into position for fusion, as they do for compatible pairs, but the process is interrupted abruptly by a cytotoxic effector cascade.

In the present study, large holes were observed in the tip cells of rejecting oozooid ampullae (Fig. 2C). Whether these were formed as part of the aborted fusion sequence (and perhaps were the means by which blood exchange leading to rejection was made) or were released endocytotic vacuoles transporting blood cells into the tunic (DeSanto, 1968) remains to be determined. In these cases, however, blood cell stasis and clumping became apparent soon after ampullar contact was established (Fig. 4A–C). This suggests that blood exchange of some kind must occur before rejection can begin, and, indeed, the first result of contact between ampullae (compatible or incompatible) appears to be tip cell alteration. Electron-microscopic examination of fusing ampullar junctions has revealed "fenestrations" in the tip cells (Katow and Watanabe, 1980). After rejections, likewise, India Ink injected into a retreating ampulla was shown to leak through the tip cells into the tunic (Taneda and Watanabe, 1982c). The results of the rejection reaction activated by mixing of allogeneic blood elements are: (1) rapid isolation of the involved structures, and (2) eventual reseparation of the allogeneic colonies.

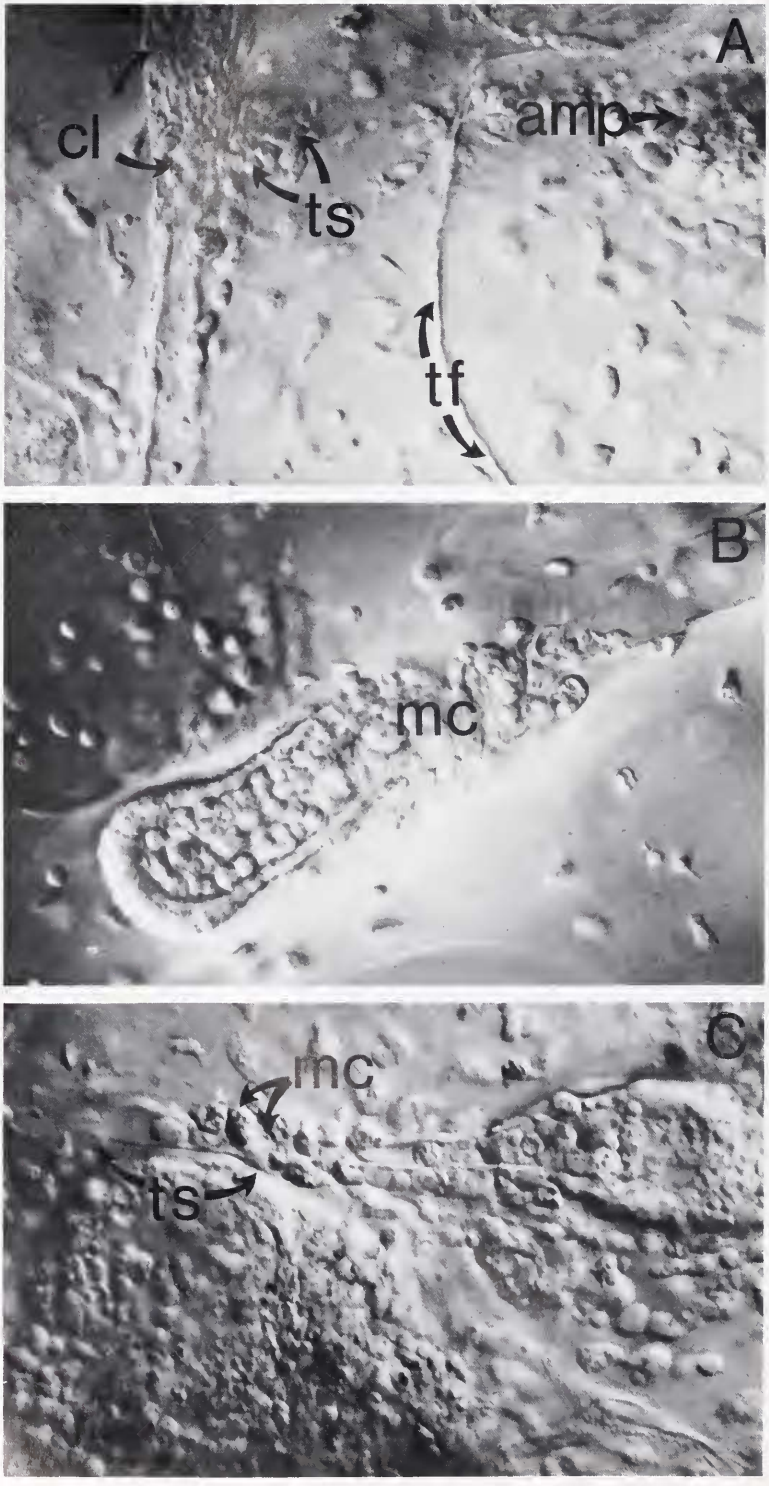
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TABLE 1

Percentages of each of four types of rejection (Types 1–4) in the paired F_1 progeny of colonies fertilized (1) by many different paternal colonies in the natural environment ("wild-fertilizations", Fig. 5, left), (2) by one paternal colony ("defined" crosses, Fig. 5, center) and (3) by self sperm ("self", Fig. 5, right)

		Rejection type			
Crosses[1]	*n* (pairs)	1	2	3	4
Wild	50	13	13	11	13
	46	13	11	9	13
	23	5	6	4	8
	50	15	14	9	12
Defined	5	1			4
	3		1		2
	8		1	7	
	11		11		
	6	6			
	16	8	5	3	
	5	1	3	1	
	5		4	1	
	9	6	3		
	6	4	2		
Self	3	3			
	2	2			
	5		5		

[1] Rejections are typed as (1) slight bleeding; (2) severe bleeding; (3) ampullar autoamputation; and (4) ampullar disintegration. For details, see text and Figure 4.

Botryllus provides one of only two known examples of genetically controlled allorecognition and response in tunicates. As shown for the solitary tunicate *Halocynthia* (Fuke, 1980; Fuke and Numakunai, 1981), allogeneic mixtures of *Botryllus* blood cells undergo rapid contact-mediated cytolysis (Scofield, in prep.). The most striking features of the *in vitro* and *in vivo* reactions between allogeneic *Botryllus* blood cells are their speed, the lack of requirement for an induction period, and the characteristic golden-brown color of the rejection lesion itself. The best clues as to cellular mechanisms for *Botryllus* alloreactivity may come from recent studies with vertebrates.

In mammals, a class of natural killer (NK) cells has been described (Herberman, 1982). Such cells have native capacities for rapid, nonimmune recognition and killing of cells of certain tumor lines, and may play a role in rejection of transplanted allogeneic blood cells (Rolstad *et al.*, 1983). Like neutrophils and monocytes, but unlike cytolytic T lymphocytes, vertebrate NK cells appear to employ reduced oxygen

FIGURE 4. Different types of cytotoxic response following a rejection reaction. A. Bleeding after partial completion of the fusion sequence. Blood cells are moving out of the "tipping" ampulla as it retreates (above right, *amp*). The site of prior tunic fusion (*tf*) and tip-to-side contact (*ts*) are marked clearly. B. Ampullar autoamputation following a rejecting tip-to-side contact. The pinching site is surrounded by morula cells (*mc*). C. Ampullar disintegration at the site of a prior tip-to-side contact (*ts*). Morula cells (*mc*) are adhered to the blood vessels of the "side" ampulla (top). ×1000.

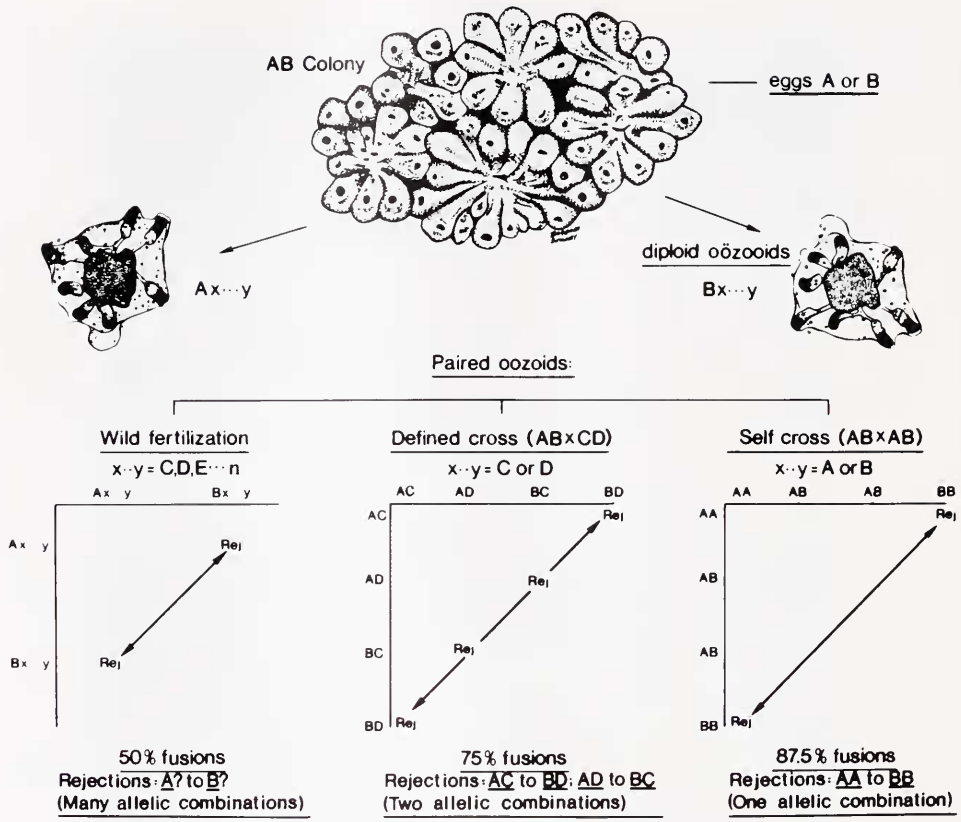

FIGURE 5. Schematic diagram showing genetic crosses performed in this study, progeny genotype ratios, and haplotypes represented in rejecting oozooid progeny pairs. Wild colonies are heterozygotic at one locus for fusibility, at which there are many alleles segregating in natural populations (50–100 in North American *Botryllus* species; Schlumpberger and Scofield, unpub.). If the mother colony is designated *AB* at the fusion locus (top), and the fertilizing sperm alleles designated *x. .y,* the oozooid progeny of any genetic crossing will be *Ax. .y* or *Bx. .y* in 1:1 proportions. *Left:* Wild fertilizations: many different fertilizing sperm (from an unknown number of paternal colonies) fertilize the *A* and *B* eggs. If rejection type is determined by fusibility haplotypes, all four rejection types should be seen in the paired offspring. *Center:* Cross-fertilizations: the maternal *AB* colony is fertilized by sperm carrying one of two fusibility alleles (*C* or *D*); rejections among the paired offspring will be *AC to BD* or *AD to BC;* therefore, one or two rejection types should be seen among the paired F_1 progeny. *Right:* Self-fertilizations: the only rejecting haplotype combination is *AA to BB;* thus, only one rejection type is expected.

intermediates in their cytolytic pathways (Roder *et al.,* 1982). We have found that mixed allogeneic *Botryllus* blood cells release both hydrogen peroxide and ferrous iron (Poenie and Scofield, in prep.), and that peroxidase appears in the tunic area around rejecting ampullae (Nynäs-McCoy, unpub.). Ascidian morula cells carry the transition metals vanadium, niobium, or iron (Goodbody, 1974; Rowley, 1982). In *Botryllus,* the morula cells contain reduced iron and sulfuric acid (Milanesi and Burighel, 1979). The morula cells are conspicuous participants in rejection lesions, where their transparent vacuoles turn a dark red-brown (Fig. 3B). Since this color reaction may reflect a change in the oxidation state of the contained iron, it is tempting to speculate that tunicate transition metals participate in allogeneic effector reactions by performing a catalytic function. All tunicates have large amounts of bound iodine

in the blood and tunic matrix (Barrington, 1975). It is interesting to note, therefore, that hydrogen peroxide, ferrous sulfate, and potassium iodide together can generate cytotoxic iodide (I ·) and hydroxyl (OH ·) radicals (Klebanoff, 1982). If tunicate metal ions initiate free radical-generating reactions, such intermediates could participate, as they may in vertebrates, in killing of bacteria or allogeneic cells. In *Botryllus,* for example, they might polymerize fibers from tunic or blood-borne precursors for clotting or encapsulation functions. Discovery of such a role(s) for tunicate transition metals might help to solve the long-standing mystery of their adaptive function (Goodbody, 1974).

Our observation of broad heterogeneity in rejection types in *Botryllus* is reminiscent of findings by Koyama and Watanabe (1982) with *Perophora sagamiensis,* where two distinct types of rejection were observed. Our studies suggest that colony specificity in *B. schlosseri* occurs on a continuum, where the time required for response varies for different pairs of interacting alleles. If the hierarchy of alleles in *Botryllus* reflects diverse thresholds for initiation of the rejection reaction, such differences might likewise explain interspecies variations noted by Koyama and Watanabe (1982) for different botryllid ascidians (see also Scofield, 1983).

The clear differences between the "acute" rejections described in this study and the "chronic" reactions occurring subsequent to fusion in some colony pairs (Mukai, 1967; Sabbadin and Zaniolo, 1979; Saito and Watanabe, 1982; Taneda and Watanabe, 1982c; Scofield, 1983) offer the intriguing possibility that in *Botryllus,* as in mammals, there may be two systems for cellular defense: the rapid, NK-like, native reaction described above, and a slower, induced response. Many different cell types appear to participate in *in vivo* or *in vitro* reactions between fully allogeneic blood cells (Scofield, in prep.). By contrast, the "delayed" response to semiallogeneic cells is significantly attenuated by X-irradiation of the recipient colony, a treatment that affects the numbers of lymphocyte-like cells (Taneda and Watanabe, 1982c).

It appears that both types of *Botryllus* allorecognition are controlled in some way by genes within the fusibility complex. The rapid, "acute" response serves a primary protective function against allogeneic invasion. Reactions between cells mixed after fusion, on the other hand, may prevent continued resource sharing between distantly related colonies that happen to share one fusibility allele. If the induction period for this response (about two weeks; Taneda and Watanabe, 1982c) reflects the time required for activation or expansion of clones of specific alloreactive cells, it may be significant that the genetics of these semiallogeneic reactions resemble those for vertebrate allograft rejection by cytotoxic T lymphocytes.

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